

ORIGINAL ARTICLE

Anti-CD52 antibody treatment in murine experimental autoimmune encephalomyelitis induces dynamic and differential modulation of innate immune cells in peripheral immune and central nervous systems

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Abstract

Anti-CD52 antibody (anti-CD52-Ab) leads to a rapid depletion of T and B cells, followed by reconstitution of immune cells with tolerogenic characteristics. However, very little is known about its effect on innate immune cells. In this study, experimental autoimmune encephalomyelitis mice were administered murine anti-CD52-Ab to investigate its effect on dendritic cells and monocytes/macrophages in the periphery lymphoid organs and the central nervous system (CNS). Our data show that blood and splenic innate immune cells exhibited significantly increased expression of MHC-II and costimulatory molecules, which was associated with increased capacity of activating antigen-specific T cells, at first day but not three weeks after five daily treatment with anti-CD52-Ab in comparison with controls. In contrast to the periphery, microglia and infiltrating macrophages in the CNS exhibited reduced expression levels of MHC-II and costimulatory molecules after antibody treatment at both time-points investigated when compared to controls. Furthermore, the transit response of peripheral innate immune cells to anti-CD52-Ab treatment was also observed in the lymphocyte-deficient SCID mice, suggesting the changes are not a direct consequence of the mass depletion of lymphocytes in the periphery. Our study demonstrates a dynamic and tissue-specific modulation of the innate immune cells in their phenotype and function following the antibody treatment. The findings of differential modulation of the microglia and infiltrating macrophages in the CNS in comparison with the innate immune cells in the peripheral organs support the CNS-specific beneficial effect of alemtuzumab treatment on inhibiting neuroinflammation in multiple sclerosis patients.

KEYWORDS

anti-CD52 antibody, EAE, innate immune cells

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INTRODUCTION

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS) and the leading cause of non-traumatic neurological disability in young adults [1]. While the exact aetiology remains unclear, MS is seen as an immune-mediated disease targeting myelin and axonal antigens [2,3]. The pathogenesis of MS is often thought to be driven by various types of immune cells including T cells, in particular due to the fact that CNS neuroinflammation can be induced in an animal model of MS via adoptive transfer of antigen-specific T cells [4,5]. Furthermore, several disease-modifying therapies targeting T-cell function or migration into the CNS have been developed for MS treatment [6–8].

One such therapy is alemtuzumab, a humanized IgG1 kappa monoclonal antibody which specifically binds to CD52, a surface glycoprotein highly expressed on T and B cells [9]. Alemtuzumab treatment induces long-lasting disease suppression in relapsing and remitting MS patients [10,11] via profound depletion of T and B lymphocytes, followed by gradual repopulation of these cells [12–14]. We previously confirmed immediate and marked depletion of lymphocytes following murine anti-CD52 antibody (anti-CD52-Ab) treatment in experimental autoimmune encephalomyelitis (EAE) mice, resulting in significantly reduced disease severity and associated CNS neuroinflammation [15]. Interestingly, the treatment does not alter the total cell number of innate monocytes, macrophages or dendritic cells (DCs) in the blood and spleen tissues [15], all of which express low levels of CD52.

While innate immune cells including monocytes, macrophages and DCs from the periphery and microglia from the CNS are crucial in the immediate response to pathogens, they are also known to play important roles in MS initiation and progression. Adoptive transfer of myelin oligodendrocyte glycoprotein (MOG) peptide primed DCs alone can induce EAE [16]. Furthermore, DC infiltration is increased within cerebrospinal fluid and demyelinating CNS lesions of MS patients [17–19]. Macrophages and monocytes are the most abundant cells present in MS lesions [20,21] and correlate with demyelination [22,23], axonal damage and degeneration [21,24]. While peripheral innate immune cells likely drive the disease process during the early phase of MS, immune reactions within the CNS involving resident microglia dominate the progressive phase of the disease [25]. Microglia are shown to respond to any external and internal insults in the CNS microenvironment and exhibit diverse reactive phenotype during the development of MS and other neuroinflammatory diseases [26,27].

Many studies have focused on the specific mechanisms by which anti-CD52-Ab depletes peripheral

lymphocytes and subsequently reduces cell infiltration into the CNS. However, very little is currently known about the precise effect of anti-CD52-Ab on DCs and macrophages in the peripheral lymphoid organs and microglial cells in the CNS.

Therefore, this study investigated the phenotypical and functional response of these innate immune cells in the short (first day post-injection) and long term (three weeks post-injection) following the treatment of EAE mice with anti-CD52-Ab. Our findings provide new insights into the immune modulatory function of alemtuzumab when it is used to treat patients with MS or other immune-mediated diseases.

MATERIALS AND METHODS

Mice and EAE induction

Naïve C57BL/6J mice were bred and maintained in the Biological Procedure Unit at the University of Strathclyde. CB17/Icr-Prkdcscid/IcrIcoCrl congenic immunodeficient mice were purchased from Charles River, UK. Female mice (9–10 weeks of age) were used in all experiments. All *in vivo* experimental procedures comply with ARRIVE guidelines and were performed under the guidelines of the UK Animals (Scientific Procedures) Act 1986 and were conducted under a Project License granted by the UK Home Office and with local ethical approval. For EAE induction, mice were immunized *s.c.* on day 0 with 100 μ l of 300 μ g MOG_{35–55} (ChinaPeptides Co., Ltd.) emulsified in complete Freund's adjuvant (CFA; Sigma) supplemented with 500 μ g *Mycobacterium tuberculosis* (BD Biosciences). Each mouse also received 250 ng pertussis toxin (PTX; Tocris Bioscience) in 100 μ l phosphate-buffered saline (PBS) injected *i.p.* on day 0 and again on day 2.

Murine anti-CD52-Ab treatment

Anti-mouse CD52 monoclonal antibody was generated as previously described [28] and provided by Genzyme. Mice were treated with either 100 μ l of PBS as a vehicle control or 10 mg/kg murine anti-CD52-Ab injected *s.c.* into the neck scruff once a day for five consecutive days. For EAE mice, animals were treated with five daily injection of anti-CD52-Ab or vehicle as described above following the onset of clinical signs of disease (Figure 1A). Mice were then killed either the day following the last injection of anti-CD52-Ab (short-term/immediate effect) or three weeks (long-term effect) after the last injection, and tissues were obtained for analysis (Figure 1A).

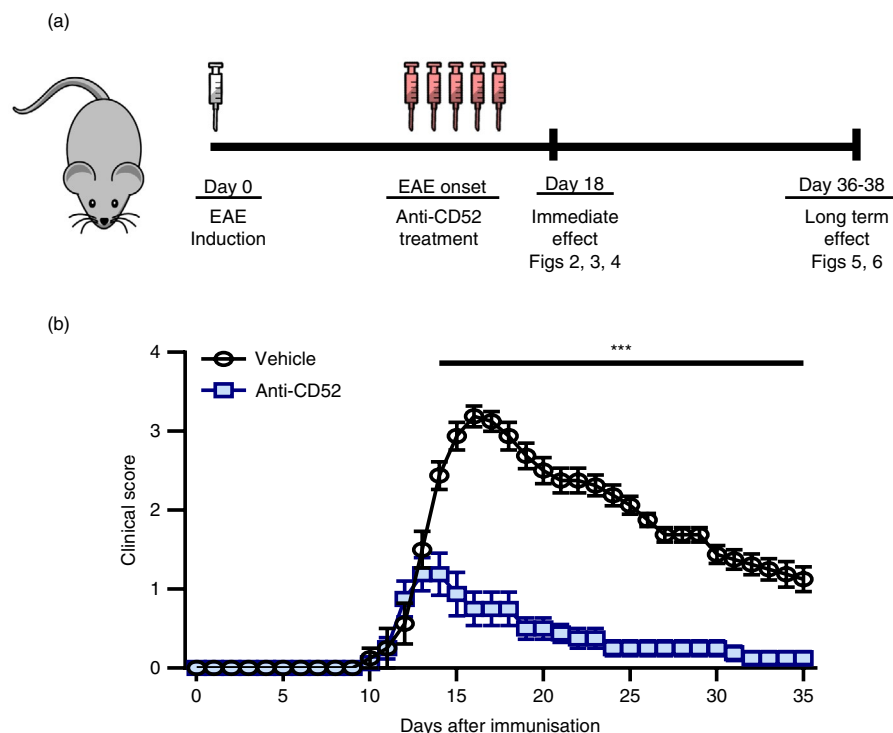


FIGURE 1 Treatment of anti-CD52-Ab in EAE mice. (A) Schematic diagram showing the treatment regime and time-points assessed. Mice were immunized with MOG₃₅₋₅₅ peptide and following onset of clinical signs of disease, treated with either vehicle or 10 mg/kg murine anti-CD52-Ab for five daily doses. At first day (immediate effect) or 3 weeks (long-term effect) following the last injection, tissues of blood, spleen and CNS were harvested for analysis. (B) EAE clinical score in mice treated with anti-CD52-Ab or vehicle, $n = 10$. Two-way ANOVA with repeated-measures and Sidak multiple comparisons test. *** $p < 0.001$

Preparation of cell suspensions

Spleen and inguinal draining lymph node (dLNs) cell preparation: mice were killed, and following PBS perfusion, tissues were removed and transferred to complete RPMI 1640 media (cRPMI; Lonza) containing 10% foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (all Sigma-Aldrich). Tissues were mashed gently using a syringe plunger top and passed through a 70- μ m cell strainer to obtain a single-cell suspension. Red blood cells in the suspension were lysed with eBioscience 10 \times RBC Lysis Buffer (Thermo Fisher Scientific), and cells were ready for further experiments.

Blood cell preparation: mice were killed and blood collected into microcentrifuge tubes containing PBS with 5 mM EDTA (Thermo Fisher Scientific). Red blood cells were then lysed as above, and cells were washed with PBS and resuspended in appropriate medium for further downstream applications.

Isolation of mononuclear cells from CNS tissue: a protocol by Grabert and McColl was followed [29]. Briefly, mice were killed and, following PBS perfusion, brains were harvested and spinal cords flushed out with PBS by hydrostatic pressure. Brain and spinal cord tissues were then enzymatically digested in HBSS (Fisher Scientific) with 2 mg/ml Collagenase D and 1 mg/ml DNase (both Sigma-Aldrich) at 37°C for 1 h. Mononuclear cells were then isolated by percoll gradient, with cells resuspended in 35% percoll (Sigma-Aldrich) and overlaid with 5 ml HBSS. Following centrifugation, the pelleted mixed CNS

cells were washed in HBSS then resuspended in appropriate medium for further applications.

Flow cytometry

Single-cell suspensions from blood, spleen or CNS tissues were added to flow cytometry tubes or 96-well U-bottom plates (0.5×10^6 cells per tube or well). To block non-specific Fc receptors, cells were resuspended in anti-mouse CD16/CD32 Fc block (eBioscience) for 10 min before incubation with the appropriate antibodies against: CD11b (#130-113-236), CD11c (#130-102-545), CD38 (#130-109-257), CD40 (#130-105-376 or #130-102-533), CD80 (#130-116-465), CD86 (#130-105-137), MHC-II (#130-107-942), all were purchased from MACS Miltenyi Biotec; and CD45 (#11-0451-82) and CD206 (#12-2061-82), all were purchased from Thermo Fisher Scientific. Viable cells were then identified by staining with the LIVE/DEAD Fixable Violet Dead Cell Stain Kit following manufacturer's guidelines (Thermo Fisher Scientific). Cells were then resuspended in 0.2–0.5 ml flow cytometry buffer and analysed using a BD FACSCanto system and BD FACSDiva software (both BD Biosciences) or Attune NxT Flow Cytometer and Attune NxT software (Thermo Fisher Scientific).

Immune cell isolations

Single-cell suspensions were prepared as described above from spleen, blood or CNS tissues. CD11b⁺ cells (CD11b

microbeads #130-049-601), CD11c⁺ cells (pan dendritic cell isolation kit #130-100-875) or CD4⁺ T cells (CD4⁺ T cell isolation kit #130-104-454) were then isolated using MACS Miltenyi Biotec kits following manufacturer's instructions. Separation was achieved using LS columns placed in the magnetic field of either MidiMACS or QuadroMACS separators attached to a MACS MultiStand. All reagents and equipment were from MACS Miltenyi Biotec.

Sample preparation for ELISA

Isolated cell populations were added to 96-well plates (1×10^5 cells per well for CNS CD11b⁺ cells; 5×10^5 cells per well for spleen CD11c⁺ cells and blood CD11b⁺ cells; 1×10^6 cells per well for spleen CD11b⁺ cells) and cultured either with media alone, or with 100 ng/ml LPS or 20 µg/ml poly(I:C) in the media at 37°C for 24 h. Supernatants were then collected and the concentrations of IL-6, IL-1β and TNF-α were determined by ELISA following manufacturer's protocols (all Thermo Fisher Scientific kits).

Co-culture of innate immune cells with T cells

Antigen-presenting innate CD11c⁺ or CD11b⁺ cells were pulsed with (+MOG) or without (−MOG) 100 µg/ml MOG₃₅₋₅₅ peptide for 4 h at 37°C/5% CO₂ and then washed three times with PBS. Meanwhile, dLNs were collected from mice at the peak of EAE severity and CD4⁺ T cells were isolated using MACS Miltenyi Biotec kits as described in section 'Immune cell isolations'. Purified CD4⁺ T cells were then cultured with washed MOG₃₅₋₅₅-pulsed or non-pulsed CD11c⁺ or CD11b⁺ cells at a ratio of 10:1 (Tc: APCs) in 96-well plates. At 72 h after the co-culture, T-cell proliferation was assessed using an MTT assay. To analyse the cytokine production by the co-cultured cells, supernatants were collected after 72 h and IL-17 and IFN-γ levels were determined by ELISA.

Phagocytosis assay

The phagocytic capacity of isolated innate immune cells was assessed using a phagocytosis assay kit and following the manufacturer's protocol (Cayman Chemicals). CD11b⁺ cells isolated from blood or spinal cord were added to flow cytometry tubes or 96-well U-bottom plates at a concentration of 1×10^5 cells per test for CNS CD11b⁺ cells and 1×10^6 cells per test for blood CD11b⁺ cells,

both in 100 µl. Latex beads-Rabbit IgG-FITC complex was added to the cell suspension at a final dilution of 1:200 and incubated at 37°C for 24 h. Cells were washed and FITC staining was analysed by flow cytometry to assess the degree of phagocytosis.

Respiratory burst assay

The respiratory burst response of isolated innate immune cells was assessed using a respiratory burst assay kit and following the manufacturer's protocol (Cayman Chemicals). CD11b⁺ cells isolated from blood or spinal cord were added to flow cytometry tubes or 96-well U-bottom plates at a concentration of 1×10^5 cells per test for CNS CD11b⁺ cells and 1×10^6 cells per test for blood CD11b⁺ cells, both in 100 µl. Cells were incubated at 37°C with dihydrorhodamine 123 (DHR 123) for 15 mins. The conversion of DHR 123 to rhodamine 123 by reactive species was analysed by flow cytometry.

Statistics

All data are shown as mean ± SEM. Data were analysed by two-tailed, unpaired Student's *t* test or two-way ANOVA with Sidak multiple comparisons tests as appropriate. A *p* value of <0.05 was considered statistically significant.

RESULTS

Anti-CD52-Ab treatment in EAE mice modulates the phenotype of peripheral innate immune cells at first day post-injection

Anti-CD52-Ab treatment significantly ameliorates EAE clinical severity (Figure 1B). We have previously demonstrated that this treatment substantially depletes both T and B lymphocytes and, to a lesser extent, NK cells but does not reduce the number of innate immune cells including DCs or monocytes/macrophages/microglia in EAE mice [15]. Here, we investigated the phenotypical status of these cells following anti-CD52-Ab treatment in EAE mice by flow cytometry (Figure 2). We firstly gated on CD11c⁺ DCs within blood and spleen cells and analysed the expression of key activation markers MHC-II, CD40, CD80 and CD86 (Figure 2B). At first day post-injection, CD11c⁺ cells from blood showed significant upregulation of CD40 ($30.6 \pm 3.1\%$ vs. $13.3 \pm 3.7\%$; $p = 0.007$) and CD86 ($28.4 \pm 2.9\%$ vs. $11.0 \pm 1.2\%$; $p < 0.0001$) relative to vehicle controls. In addition, the percentage of splenic CD11c⁺ cells

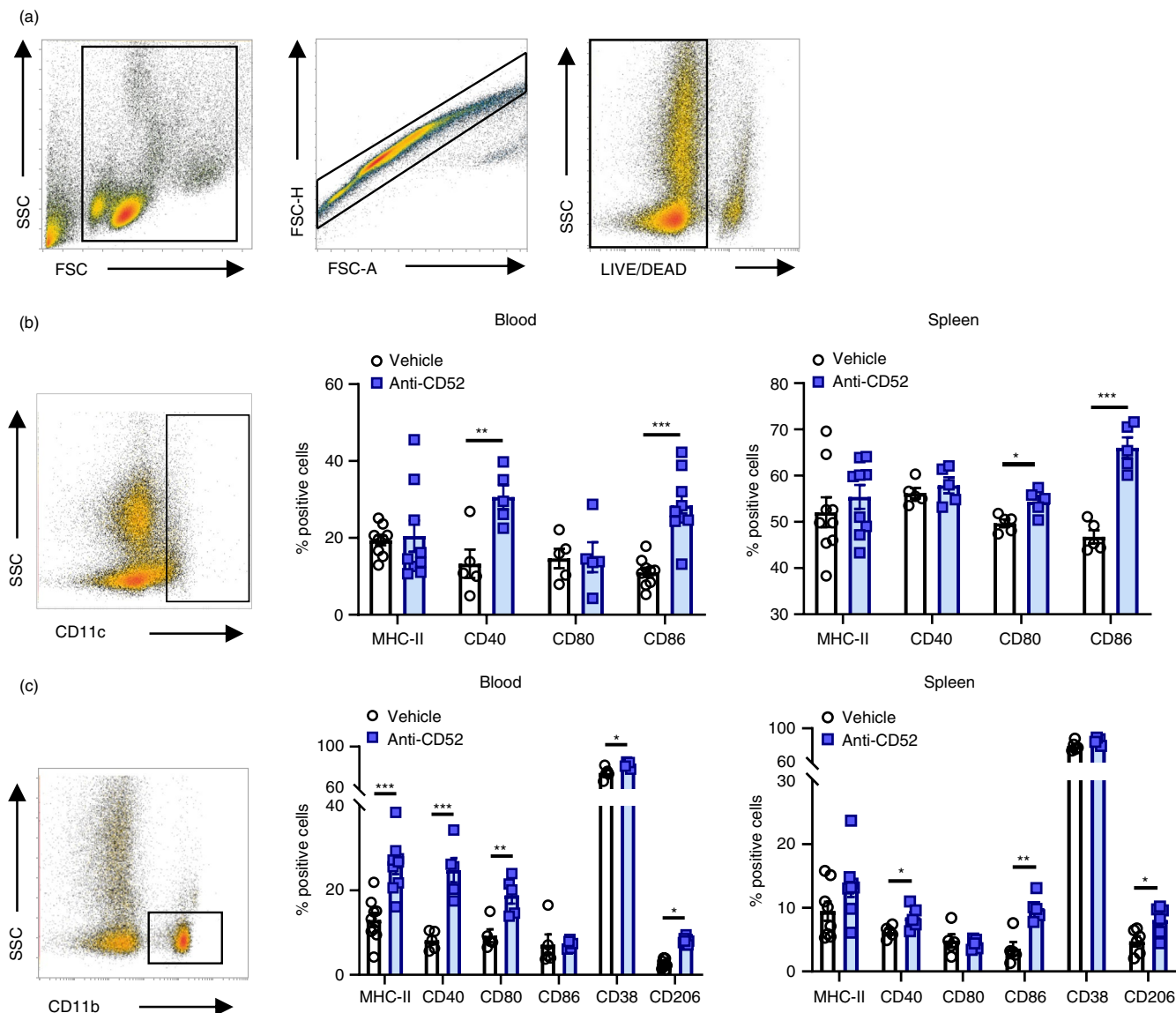


FIGURE 2 Effect of anti-CD52-Ab treatment on the phenotype of periphery innate immune cells in EAE mice at first day post-injection. Single-cell suspensions were obtained from blood and spleen tissue of anti-CD52-Ab or vehicle-treated EAE mice at first day after the final dose injection. Cells were stained for various surface markers and analysed by flow cytometry. (A) Flow cytometry gating strategy. (B) Blood and spleen CD11c⁺ cells were analysed for expression of MHC-II ($n = 9$), CD40 ($n = 5$), CD80 ($n = 5$) and CD86 ($n = 9$ blood; $n = 5$ spleen). (C) Blood and spleen CD11b⁺ cells were analysed for expression of MHC-II ($n = 8$), CD40 ($n = 5$), CD80 ($n = 5$), CD86 ($n = 5$), CD38 ($n = 5$) and CD206 ($n = 9$ blood; $n = 7$ spleen). Unpaired two-tailed Student's *t* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

expressing CD80 ($54.4 \pm 1.2\%$ vs. $49.7 \pm 0.8\%$; $p = 0.012$) and CD86 ($66.0 \pm 2.2\%$ vs. $46.8 \pm 1.4\%$; $p < 0.0001$) was also significantly increased in antibody-treated mice compared with vehicle controls. All other markers remained comparable between the two groups.

We next looked at the expression of these markers, as well as the common phenotype identifiers CD38 and CD206, on circulating monocytes and splenic macrophages. We gated on CD11b⁺ cells, before using side scatter to distinguish between these cells and neutrophils (Figure 2C). The percentage of blood monocytes expressing MHC-II ($25.9 \pm 2.1\%$ vs. $13.0 \pm 1.7\%$;

$p = 0.0002$), CD40 ($24.8 \pm 2.9\%$ vs. $8.1 \pm 1.0\%$; $p = 0.0006$), CD80 ($18.8 \pm 2.0\%$ vs. $9.3 \pm 1.5\%$; $p = 0.005$), CD38 ($83.0 \pm 1.3\%$ vs. $75.1 \pm 2.4\%$; $p = 0.021$) and CD206 ($8.1 \pm 0.3\%$ vs. $3.2 \pm 0.4\%$; $p < 0.0001$) were all significantly increased in EAE mice receiving anti-CD52-Ab compared with controls (Figure 1D). In the spleen, the percentage of macrophages expressing CD40 ($8.5 \pm 0.8\%$ vs. $6.2 \pm 0.4\%$; $p = 0.041$), CD86 ($9.9 \pm 0.9\%$ vs. $3.6 \pm 1.1\%$; $p = 0.002$) and CD206 ($8.1 \pm 0.9\%$ vs. $4.7 \pm 0.8\%$; $p = 0.021$) was significantly upregulated in anti-CD52-Ab treated EAE mice versus mice treated with vehicle alone.

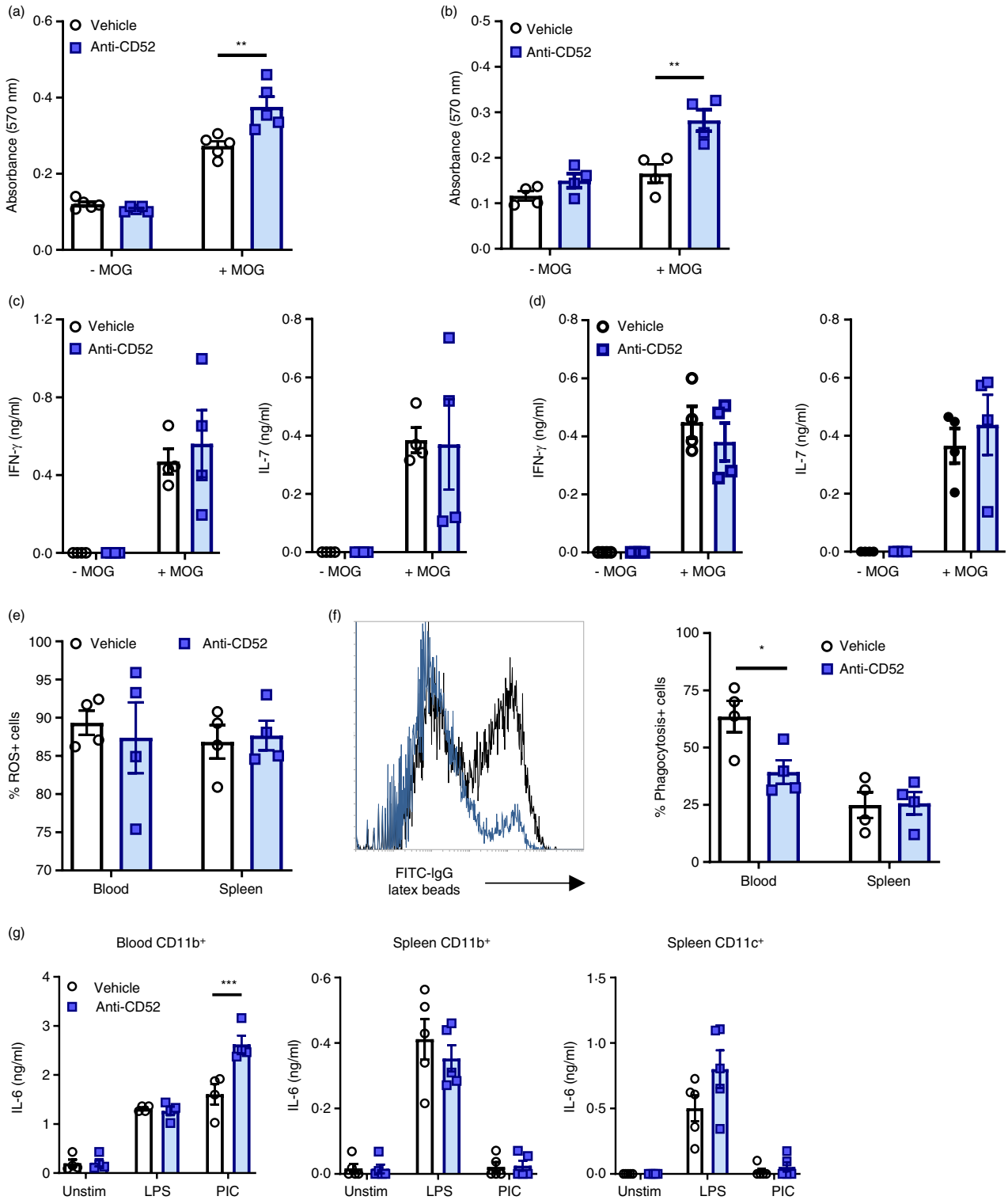


FIGURE 3 Effect of anti-CD52-Ab treatment on the function of periphery innate immune cells in EAE mice at first day post-injection. CD11c⁺ cells were isolated from spleen and CD11b⁺ cells isolated from spleen or blood of anti-CD52-Ab or vehicle-treated EAE mice at first day after the final treatment dose. (A) Spleen CD11c⁺ cells or (B) blood CD11b⁺ cells were pulsed with (+MOG) or without (-MOG) MOG₃₅₋₅₅ for 4 h and then thoroughly washed before 72-h co-culture with CD4⁺ T cells isolated from lymph nodes of peak stage EAE mice. T-cell proliferation was examined by MTT assay (A and B). Supernatants were also collected and IL-17 and IFN- γ production determined by ELISA for co-culture with (C) spleen CD11c⁺ cells or (D) blood CD11b⁺ cells. Two-way ANOVA with Sidak multiple comparisons test. (E) Respiratory burst activity of CD11b⁺ isolated cells. (F) Phagocytosis of FITC-coated beads by CD11b⁺-isolated cells. Unpaired two-tailed Student's *t* test. (G) Cells were left unstimulated or were stimulated with LPS or Poly(I:C)(PIC) for 24 h and cytokine production determined by ELISA. Two-way ANOVA with Sidak multiple comparisons test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001

Anti-CD52-Ab treatment in EAE mice alters the function of peripheral innate immune cells at first day post-injection

Having observed clear phenotype changes in peripheral CD11c⁺ and CD11b⁺ cells in EAE mice following anti-CD52-Ab treatment, we next sought to determine whether this translated to differences in cell function. We isolated splenic CD11c⁺ cells or CD11b⁺ cells from blood and spleens of EAE mice at first day after the final injection of anti-CD52-Ab or vehicle and analysed various key functions performed by innate immune cells. First, we evaluated the antigen presentation capability of DCs and macrophages by pulsing the cells with or without MOG₃₅₋₅₅ before co-culturing them with MOG₃₅₋₅₅ specific T cells and measuring proliferation by MTT assay. Our data show that proliferation of T cells showed no difference between antibody and vehicle-treated EAE mice spleen CD11c⁺ (Figure 3A) or circulating CD11b⁺ (Figure 3B) cells when they were pre-pulsed without MOG (–MOG). However, when co-cultured with pre-MOG-pulsed (+MOG) CD11c⁺ cells, T cells exhibited significantly increased proliferation when the co-cultured innate cells were isolated from the anti-CD52-Ab-treated EAE mice compared with the cells of control mice (Figure 3A; 0.38 ± 0.03 vs. 0.27 ± 0.01 ; $p = 0.001$). This was also true for CD11b⁺ cells (Figure 3B; 0.29 ± 0.02 vs. 0.17 ± 0.02 ; $p = 0.004$). We next examined T-cell cytokine production. While co-culture of T cells with innate immune cells not pre-pulsed with MOG had undetectable levels of cytokine production, T cells co-cultured with MOG₃₅₋₅₅-pulsed CD11c⁺ cells (Figure 3C) or CD11b⁺ cells (Figure 3D) produced high levels of IL-17 and IFN- γ . However, there was no difference between the cultures of innate immune cells collected from vehicle or anti-CD52-Ab-treated EAE mice.

We next analysed the respiratory burst and phagocytosis capacities of CD11b⁺ cells isolated from the blood and spleen of anti-CD52-Ab or vehicle-treated EAE mice. We observed no change in the percentage of cells which are ROS⁺ in CD11b⁺ cells from blood or spleen between anti-CD52-Ab treated and vehicle controls (Figure 3E). We also detected no difference in the level of ROS being produced as assessed by mean fluorescence intensity (data not shown).

However, the phagocytosis capacity of CD11b⁺ cells isolated from blood of anti-CD52-Ab treated EAE mice was significantly reduced ($39.3 \pm 5.1\%$ vs. $63.5 \pm 6.2\%$; $p = 0.021$) compared with the cells of vehicle-treated EAE mice (Figure 3F), while there was no change between the two groups in phagocytosis by CD11b⁺ cells isolated from the spleen. Finally, we stimulated CD11b⁺ cells isolated from blood or spleen or splenic CD11c⁺ cells with

LPS or Poly (I:C) and analysed production of IL-6, TNF- α and IL-1 β by ELISA (Figure 3G). Blood CD11b⁺ cells from anti-CD52-Ab-treated EAE mice produced significantly increased levels of IL-6 (2.6 ± 0.2 vs. 1.6 ± 0.2 ng/ml; $p < 0.0001$) in response to Poly (I:C) stimulation relative to the control mice. Spleen CD11b⁺ and CD11c⁺ cells produced comparable levels of IL-6 in both antibody- and vehicle-treated EAE groups. The levels of TNF- α production by these innate immune cells following both LPS and Poly (I:C) stimulation were also similar between the two groups of EAE mice (data not shown). Levels of IL-1 β were below the limit of detection for the assay. It should be noted that we also attempted to isolate CD11c⁺ cells from blood samples but due to extremely low cell counts further analysis was not possible here without using excessive numbers of mice.

Anti-CD52-Ab treatment in EAE mice modulates the phenotype and function of CNS microglia and infiltrating macrophages at first day post-injection

Having established that anti-CD52-Ab treatment in EAE mice had an effect on the phenotype and function of innate immune cells in the peripheral lymphoid organs at first day post-injection, we next studied the expression of MHC-II and various costimulatory molecules on CNS resident microglia and infiltrating macrophages, identified as CD11b⁺ CD45^{lo} and CD11b⁺ CD45^{hi}, respectively (Figure 4A), in EAE mice receiving anti-CD52-Ab or vehicle. In stark contrast to what was observed in the blood and spleen, levels of many markers tested were dramatically reduced following the antibody treatment. For CNS resident microglia (Figure 4B), there was a significant decrease in the percentage of cells expressing MHC-II ($16.1 \pm 2.0\%$ vs. $43.3 \pm 4.2\%$; $p < 0.0001$), CD40 ($6.1 \pm 0.6\%$ vs. $21.4 \pm 2.9\%$; $p = 0.0009$), CD80 ($5.5 \pm 0.7\%$ vs. $17.3 \pm 1.6\%$; $p = 0.0001$) and CD86 ($6.0 \pm 1.5\%$ vs. $19.7 \pm 2.5\%$; $p = 0.001$) in EAE mice treated with anti-CD52-Ab compared with vehicle controls. Similarly, the percentage of infiltrated macrophages (Figure 4C) expressing MHC-II ($18.6 \pm 2.4\%$ vs. $52.0 \pm 2.6\%$; $p < 0.0001$) and CD80 ($30.9 \pm 3.4\%$ vs. $55.8 \pm 2.5\%$; $p = 0.0003$) was significantly reduced in the CNS tissues of antibody-treated EAE mice relative to vehicle-treated EAE mice. There was no difference between the two groups in the percentage of cells expressing CD40 on macrophages, as well as CD206 on either microglia or macrophage populations.

Next, we examined the function of these CD11b⁺ innate immune cells in the CNS. We firstly analysed their respiratory burst and phagocytosis capacities. No difference was observed in the percentage of cells which were

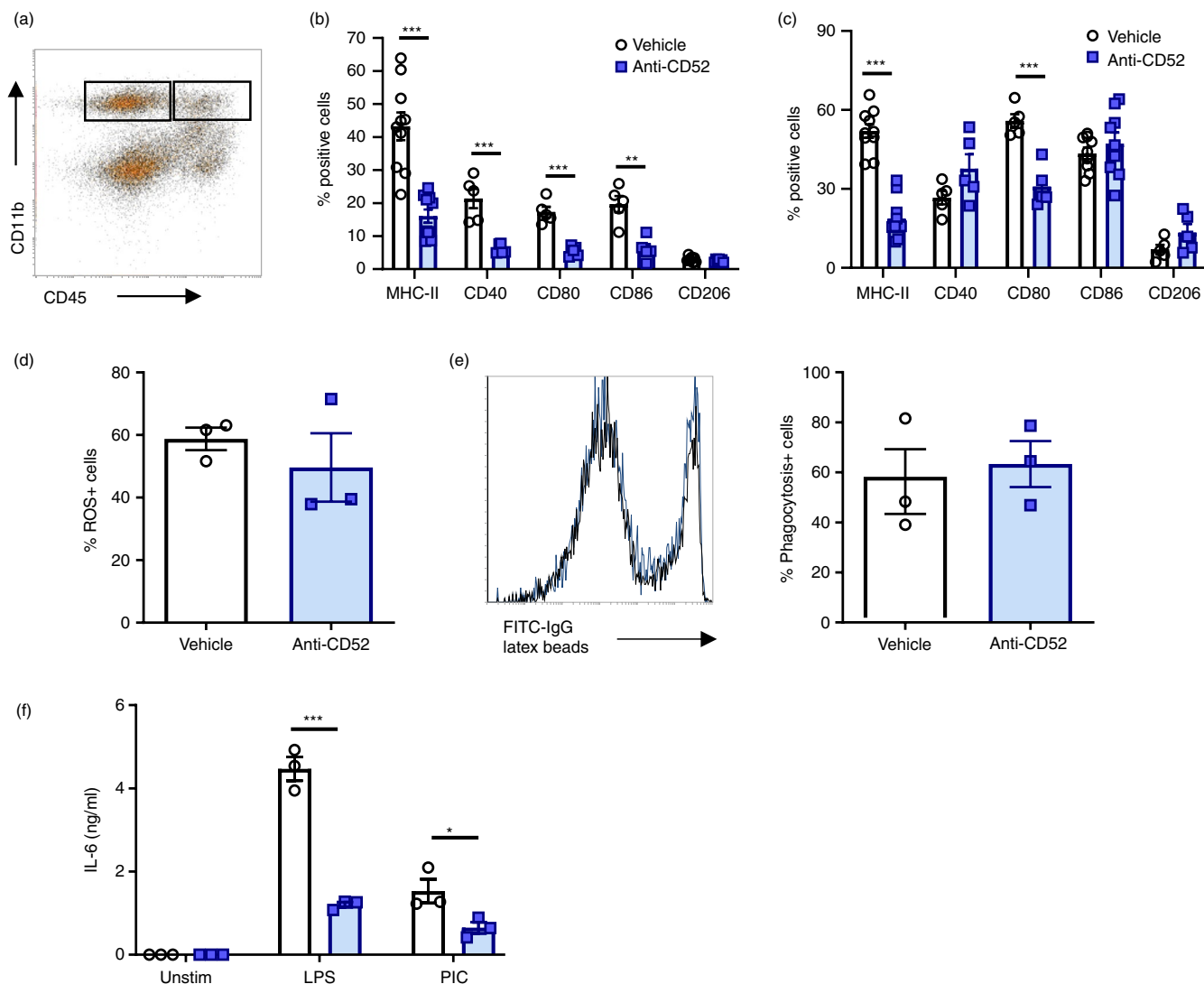


FIGURE 4 Effect of anti-CD52-Ab treatment on the phenotype and function of CNS innate immune cells in EAE mice at first day post-injection. Single-cell suspensions were obtained from CNS (brain and spinal cord) tissue of anti-CD52-Ab or vehicle-treated EAE mice at first day after the final treatment dose. Cells were stained for various surface markers and analysed by flow cytometry. (A) Flow cytometry gating strategy. (B) CD11b⁺ CD45^{lo/neg} resident microglia and (C) CNS CD11b⁺ CD45^{hi} infiltrating macrophages and were analysed for expression of MHC-II (*n* = 10), CD40 (*n* = 5), CD80 (*n* = 5), CD86 (*n* = 5 microglia; *n* = 9 macrophages) and CD206 (*n* = 7 microglia; *n* = 5 macrophages). Unpaired two-tailed Student's *t* test. CD11b⁺ cells were isolated from CNS tissue of anti-CD52-Ab or vehicle-treated mice at first day after the final treatment dose. (D) Respiratory burst activity of CD11b⁺-isolated cells. (E) Phagocytosis of FITC-coated beads by CD11b⁺ isolated cells. Unpaired two-tailed Student's *t* test. (F) Cells were left unstimulated or were stimulated with LPS or Poly(I:C)(PIC) for 24 h and cytokine production determined by ELISA. Two-way ANOVA with Sidak multiple comparisons test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001

ROS⁺ between anti-CD52-Ab-treated and vehicle controls (Figure 4D). We also detected no difference in the level of ROS being produced as assessed by mean fluorescence intensity (data not shown). Similarly, there was no difference in the percentage of phagocytosis⁺ cells between CD11b⁺ cells isolated from anti-CD52-Ab or vehicle-treated EAE mice (Figure 4E). Next CNS CD11b⁺ cells were stimulated with TLR agonists LPS or Poly (I:C), and production of IL-6, TNF-α and IL-1β was analysed by ELISA. Our data show that IL-6 production by CNS CD11b⁺ cells isolated from antibody-treated EAE mice was significantly decreased

when compared to the cells of vehicle-treated EAE mice, in response to both LPS (1.2 ± 0.1 vs. 4.5 ± 0.3 ng/ml; *p* < 0.0001) and Poly (I:C) stimulation (0.6 ± 0.1 vs. 1.5 ± 0.3 ng/ml; *p* = 0.012) (Figure 4F). However, similar levels of TNF-α production by the cells were observed between the two groups of EAE mice following stimulation with LPS or Poly (I:C) (data not shown). Levels of IL-1β were below the limit of detection for the assay. We also attempted to study the proliferation function of CNS CD11c⁺ cells; however, it was not successful possibly due to very low number of cells isolated (data not shown).

Anti-CD52-Ab treatment changes its effect on the phenotype of the peripheral but not the CNS innate immune cells at 3 weeks post-injection

We next sought to ascertain whether the altered expression of phenotype markers of innate immune cells in EAE mice with anti-CD52-Ab treatment remains over an extended period. EAE mice were injected with anti-CD52-Ab or vehicle as before, at 3 weeks after the last

injection, innate immune cells isolated from spleen, blood and CNS tissues were analysed by flow cytometry (Figure 5). Our data show that following anti-CD52-Ab treatment, the percentage of blood CD11c⁺ cells expressing CD40 ($5.1 \pm 0.6\%$ vs. $10.2 \pm 1.0\%$; $p = 0.0006$) and splenic CD11c⁺ cells expressing MHC-II ($20.9 \pm 1.0\%$ vs. $28.7 \pm 0.9\%$; $p < 0.0001$) was significantly decreased compared with vehicle-treated control mice (Figure 5A). Similarly, the percentage of splenic CD11b⁺ cells expressing MHC-II ($9.3 \pm 0.9\%$

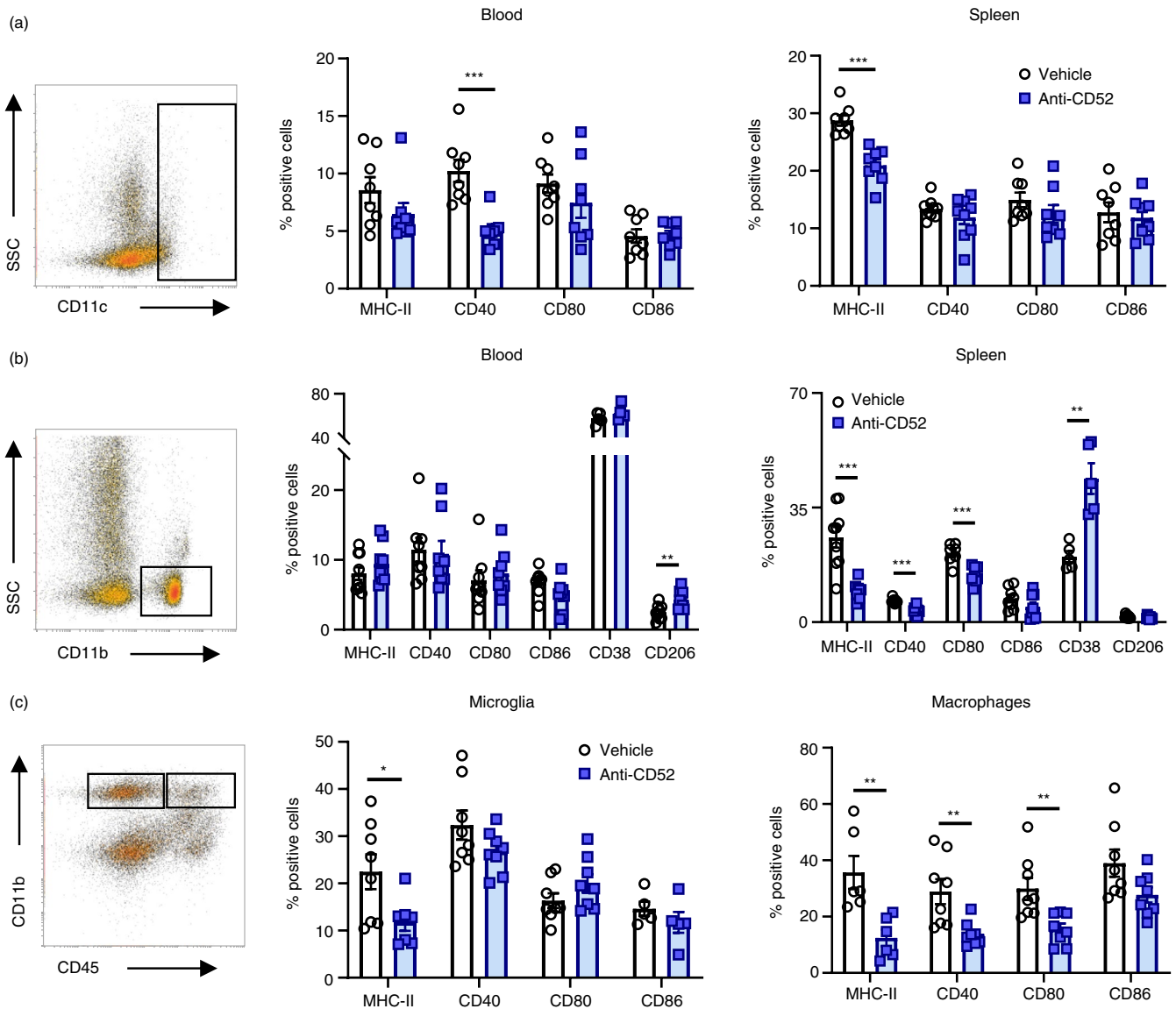


FIGURE 5 Effect of anti-CD52-Ab treatment on the phenotype of periphery and CNS innate immune cells in EAE mice at three weeks post-injection. Single-cell suspensions were obtained from blood, spleen and CNS (brain and spinal cord) tissue of anti-CD52-Ab or vehicle-treated mice three weeks after the final treatment dose. Cells were stained for various surface markers and analysed by flow cytometry. (A) Blood and spleen CD11c⁺ cells were analysed for expression of MHC-II ($n = 9$), CD40 ($n = 5$), CD80 ($n = 5$) and CD86 ($n = 9$ blood; $n = 5$ spleen). (B) Blood and spleen CD11b⁺ cells were analysed for expression of MHC-II ($n = 8$), CD40 ($n = 5$), CD80 ($n = 5$), CD86 ($n = 5$), CD38 ($n = 5$) and CD206 ($n = 9$ blood; $n = 7$ spleen). (C) CNS CD11b⁺ CD45^{hi}-infiltrating macrophages and CD11b⁺ CD45^{lo/neg} resident microglia were analysed for expression of MHC-II ($n = 10$), CD40 ($n = 5$), CD80 ($n = 5$), CD86 ($n = 5$ microglia; $n = 9$ macrophages) and CD206 ($n = 7$ microglia; $n = 5$ macrophages). Unpaired two-tailed Student's *t* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

vs. $25.9 \pm 3.1\%$; $p < 0.0001$), CD40 ($3.7 \pm 0.4\%$ vs. $6.5 \pm 0.3\%$; $p < 0.0001$) and CD80 ($14.4 \pm 0.9\%$ vs. $21.0 \pm 1.1\%$; $p = 0.0005$) in EAE mice treated with anti-CD52-Ab was significantly decreased relative to cells of vehicle-treated controls (Figure 5B). In contrast, the percentages of CD206 expressing cells out of total blood CD11b⁺ cells ($4.3 \pm 0.4\%$ vs. $2.5 \pm 0.3\%$; $p = 0.005$) and CD38 expressing cells out of total splenic CD11b⁺ cells ($43.9 \pm 4.7\%$ vs. $20.0 \pm 1.7\%$; $p = 0.001$) were significantly increased in antibody-treated EAE mice compared with control group mice (Figure 5B).

In the CNS tissues of EAE mice at 3 weeks post-injection, CD11b⁺ CD45^{lo} microglia and CD11b⁺ CD45^{hi}-infiltrating macrophages maintained similar expression profiles to that was observed at first day post-injection. In antibody-treated EAE mice, the percentage of MHC-II⁺ microglia ($11.8 \pm 1.9\%$ vs. $22.5 \pm 3.7\%$; $p = 0.028$) was significantly decreased, as was the percentage of macrophages within the CNS that were MHC-II⁺ ($12.4 \pm 2.9\%$ vs. $35.7 \pm 5.9\%$; $p = 0.005$), CD40⁺ ($13.8 \pm 1.5\%$ vs. $28.9 \pm 4.4\%$; $p = 0.006$) and CD80⁺ ($15.6 \pm 1.9\%$ vs. $29.9 \pm 3.9\%$; $p = 0.005$) compared with EAE mice receiving vehicle only (Figure 5C).

Anti-CD52-Ab treatment does not change the function of peripheral or CNS monocyte/macrophages at three weeks post-injection

We further assessed the long-term effects of anti-CD52-Ab treatment on the function of innate immune cells in EAE mice at 3 weeks after treatment. Our data show that there was no difference in CD4⁺ T cell proliferation when co-cultured with MOG₃₅₋₅₅ peptide-pulsed (+MOG) splenic CD11c⁺ cells (Figure 6A) or blood CD11b⁺ cells (Figure 6B) isolated from either vehicle or anti-CD52-Ab-treated EAE mice. Furthermore, while co-culture of T cells with innate immune cells not pre-pulsed with MOG (−MOG) had low or undetectable levels of antigen-specific cytokine production, T cells co-cultured with MOG₃₅₋₅₅-pulsed innate immune cells produced high levels of IL-17 and IFN-γ (Figure 6C, D) but with no difference between vehicle or anti-CD52-Ab-treated EAE mice for both CD11c⁺ and CD11b⁺ cells. We also assessed the phagocytotic capabilities of innate immune cells isolated from blood, spleen or CNS and observed no differences between antibody- and vehicle-treated EAE groups (Figure 6E). Finally, isolated cells were cultured with Poly (I:C) or LPS for 24 h, our results showed no difference in the levels of IL-6 production between cells from anti-CD52-Ab or vehicle-treated EAE mice in all cell types tested (Figure 6F).

Anti-CD52-Ab treatment induces phenotypic changes in monocyte/macrophages in naïve and SCID mice

To determine whether the changes in innate cell phenotype occur only in the presence of an inflammatory response such as in our EAE model, naïve mice were treated with five daily injection of anti-CD52-Ab antibody or vehicle as in EAE mice. Blood, spleen or CNS tissues were collected and analysed either 1 day or 3 weeks after the last injection. Our data of innate immune cells in the blood and spleen show a similar pattern of changes to that observed in EAE mice, with expression of several of the markers on monocytes/macrophages upregulated immediately after anti-CD52-Ab treatment (Figure S1), followed by a reduction or no change of many of these markers at 3 weeks later (Figure S2). No phenotypical changes were observed in microglia population in the CNS of these naïve mice at either time-point.

To understand whether the phenotypical changes observed in innate cells following anti-CD52-Ab treatment is the result of a direct effect of the antibody or a secondary consequence of T- and B-cell depletion, SCID mice were treated with anti-CD52-Ab or vehicle for 5 days. At one day after the last injection, phenotype of innate immune cells was analysed using flow cytometry. We firstly gated on CD11c⁺ DCs within spleen and blood (Figure 7A). Following anti-CD52-Ab treatment, CD11c⁺ cells from spleen but not blood showed significant upregulation of CD40 ($30.6 \pm 2.6\%$ vs. $9.7 \pm 1.2\%$; $p < 0.0001$) and CD86 ($25.8 \pm 3.9\%$ vs. $1.0 \pm 0.1\%$; $p < 0.0001$) relative to vehicle controls. We next gated on CD11b⁺ cells (Figure 7B). In the spleen, the percentage of CD11b⁺ expressing CD40 ($8.7 \pm 0.7\%$ vs. $3.3 \pm 0.9\%$; $p = 0.007$), CD86 ($16.4 \pm 2.5\%$ vs. $1.4 \pm 0.4\%$; $p < 0.0001$) and CD206 ($6.6 \pm 1.1\%$ vs. $1.7 \pm 0.6\%$; $p = 0.02$) was significantly upregulated in antibody-treated SCID mice versus animals received vehicle alone. Furthermore, the percentage of blood CD11b⁺ expressing CD40 ($13.6 \pm 3.0\%$ vs. $5.4 \pm 0.7\%$; $p < 0.0001$) was significantly increased in EAE mice receiving anti-CD52-Ab compared with controls. All other markers tested remained comparable between the two groups.

DISCUSSION

Studies so far with anti-CD52-Ab treatment in EAE models have primarily focused on its effect on T and B lymphocytes due to the known profound depletion of these cells by the antibody, as well as the important role T and B cells play in the development of MS. However, much less is known about the effect of the antibody on innate immune cells. We have previously shown that anti-CD52-Ab

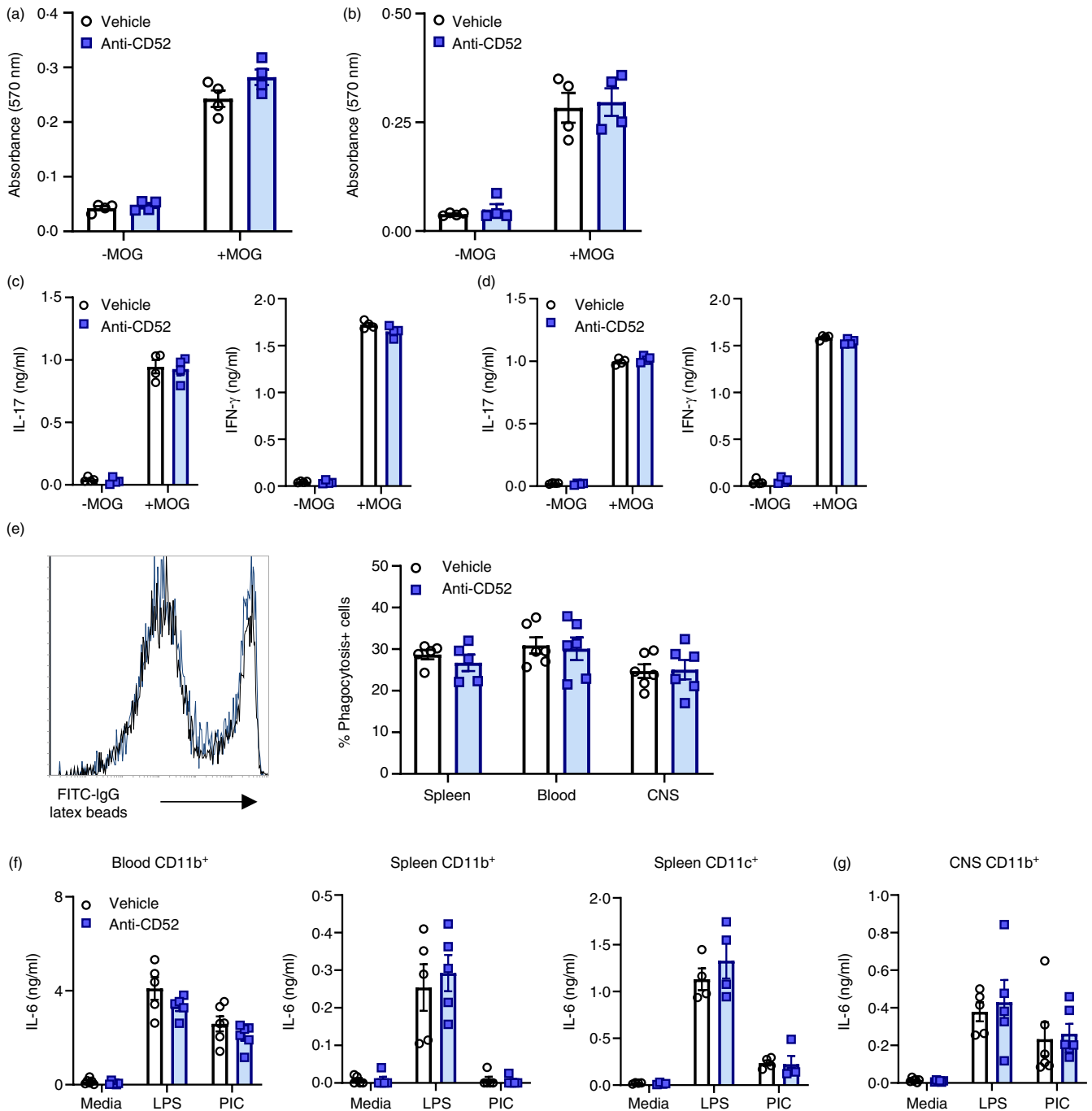


FIGURE 6 Effect of anti-CD52-Ab treatment on the function of periphery and CNS innate immune cells in EAE mice at three weeks post-injection. CD11c⁺ cells were isolated from spleen and CD11b⁺ cells isolated from spleen, blood or CNS of anti-CD52-Ab or vehicle-treated mice three weeks after the final treatment dose. (A) Spleen CD11c⁺ cells or (B) blood CD11b⁺ cells were pulsed with (+MOG) or without (-MOG) MOG₃₅₋₅₅ for 4 h before 72-h co-culture with CD4⁺ T cells isolated from the lymph nodes of peak stage EAE mice, and T-cell proliferation was examined by MTT assay. Supernatant was also collected and IL-17 and IFN- γ production determined by ELISA for co-culture with (C) spleen CD11c⁺ cells or (D) blood CD11b⁺ cells. Two-way ANOVA with Sidak multiple comparisons test. (E) Phagocytosis of FITC-coated beads by CD11b⁺ isolated cells. Unpaired two-tailed Student's *t* test. (F, G) Cells were left unstimulated or were stimulated with LPS or Poly(I:C)(PIC) for 24 h and cytokine production determined by ELISA. Two-way ANOVA with Sidak multiple comparisons test

does not deplete DCs, macrophages, monocytes or CNS resident cells [15], which are key components of the inflammatory response in EAE/MS [16,18,20,30]. Because of the lymphocyte depletion following alemtuzumab

treatment, the role of the innate immune system in the defence against infection and other immune-mediated disease is likely to be more critical in patients. Therefore, it is fundamentally important to understand the impact of

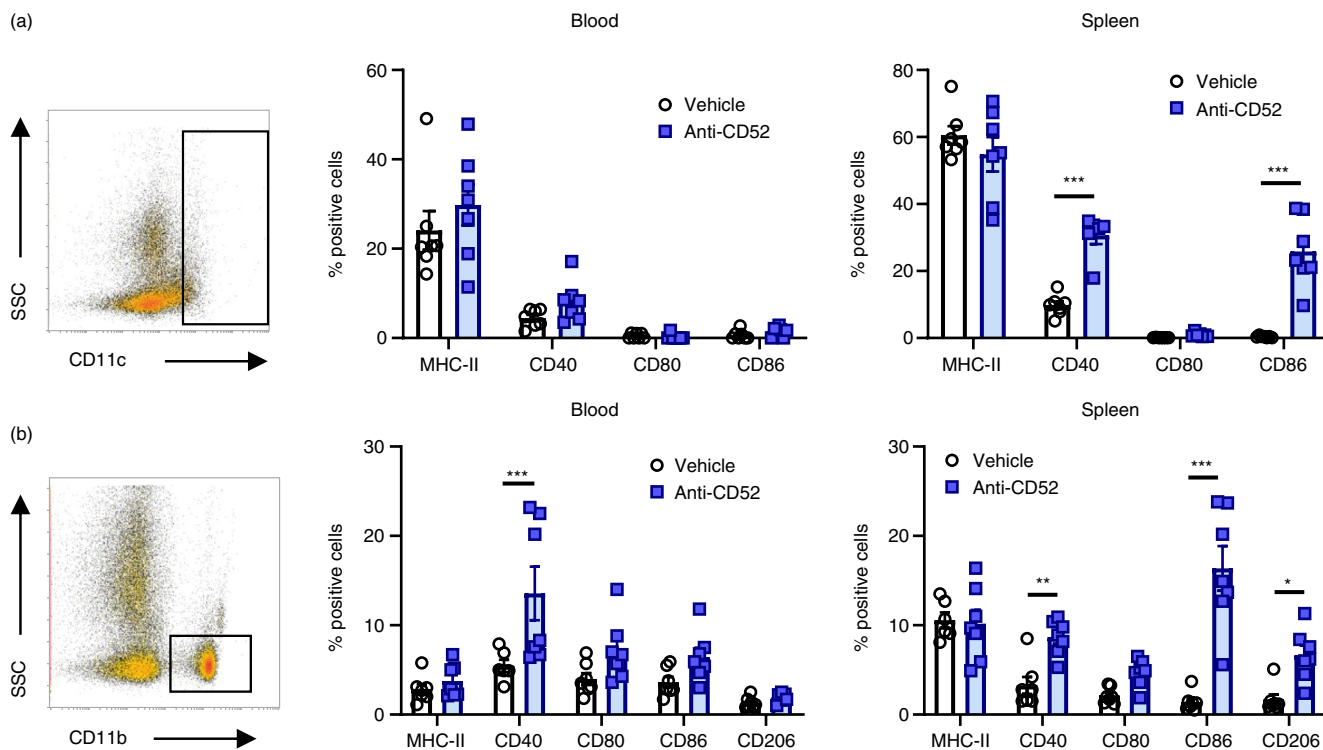


FIGURE 7 Effect of anti-CD52-Ab treatment on the function of periphery and CNS innate immune cells in SCID mice at first day post-injection. SCID mice were treated with either vehicle or 10 mg/kg murine anti-CD52-Ab-Ab for five consecutive days. Single-cell suspensions were then obtained from blood and spleen tissues first day after the final treatment dose. Cells were stained for various surface markers and analysed by flow cytometry. (A) Blood and spleen CD11c⁺ cells were analysed for expression of MHC-II, CD40, CD80 and CD86 (all *n* = 7). (B) Blood and spleen CD11b⁺ cells were analysed for expression of MHC-II, CD40, CD80, CD86, and CD206 (all *n* = 7). Unpaired two-tailed Student's *t* test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001

the treatment on innate immune cells in patients. In this study, we investigated the effect of anti-CD52-Ab treatment in EAE on the phenotype and function of DCs and monocyte/macrophage in the blood, spleen and CNS.

While treatment of EAE with anti-CD52-Ab did not change the overall number of DCs and monocytes/macrophages in the periphery [15], data from this study revealed a change of cell phenotype and function. Their expression of a variety of important markers associated with cell activation and function, e.g. MHC-II, CD40, CD80, CD86, CD38 and CD206, was significantly increased at first day after the last dose of antibody injection. The findings indicate an immediate response of the innate immune cells in the peripheral lymphoid organs to the anti-CD52-Ab treatment, and the response is likely to be tissue-specific as the changes between splenocytes and blood cells were not consistent. Functional characteristics of monocyte/macrophage are often closely associated with the cell phenotype [31]. Indeed, circulating CD11b⁺ cells isolated from anti-CD52-Ab-treated EAE mice, which had higher expression of MHC-II and costimulatory molecules CD40 and CD80, induced significantly increased proliferation of antigen-specific CD4⁺ T cells when compared to the CD11b⁺ cells of vehicle-treated EAE mice. The data suggest

an increased signal to T cells during antigen presentation. Increased levels of IL-6 production by the CD11b⁺ cells of anti-CD-52-Ab-treated mice in response to the TLR3 agonist Poly (I:C) also supports the functional change of these cells as APCs. The phagocytotic capacity of circulating CD11b⁺ cells of anti-CD52-Ab treated EAE mice was significantly decreased relative to vehicle controls, which agrees with the well-established knowledge that during the maturation process of APCs such as DC, matured cells often exhibit reduced levels of antigen capturing but have increased capacity of stimulating lymphocytes. While we agree with Turner et al. [32] that alemtuzumab treatment had minimal impact on the number of innate immune cells, our findings indicate an important phenotypic and functional alternation, albeit transit, of these circulating and splenic CD11b⁺ cells which differs from their findings of little functional impact on the isolated peritoneal CD11b⁺ cells in mice [32]. The disagreement may be explained by the phenotypic diversity of CD11b⁺ cells in different tissues [33]. Other experimental difference between the studies also makes it difficult to have a direct comparison. While Turner et al. used human CD52 transgenic mice with one dose of 1 mg/kg alemtuzumab, non-transgenic mice were used in our study with five doses of

10 mg/kg murine anti-CD52-Ab antibody which matches the treatment regime of MS patients.

The heterogeneity of monocyte and macrophage has been well documented [34]. Previously, CD38 expression has been used as a so-called 'M1' macrophage marker while CD206 identifies 'M2' macrophages [35–38]. However, it is difficult to accurately categorize these cells by the expression of these surface markers only as the cells are well known to display a large degree of plasticity resulting in a broad spectrum of possible phenotypical states [39,40]. The increased percentage of both CD38⁺ and CD206⁺ cells in the CD11b⁺ population of antibody-treated mice highlights the complexity to define these cells under the specific groups previously described such as M1, M2a, and M2b. Emerging evidence suggests that macrophage populations often display a mix of markers or characteristics associated with both M1 and M2 [38,41–43], supporting our findings here and further emphasising the necessity for robust functional analysis to fully understand the myriad potential macrophage phenotypes beyond M1 and M2.

Phenotypic and functional heterogeneity of monocyte/macrophage subsets is further supported by the differential response of peripheral and CNS innate cells to the anti-CD52-Ab treatment. In complete contrast to the increased expression of MHC-II and/or costimulatory markers observed in the blood and spleen innate cells, their expression on microglia and CNS infiltrating macrophages was significantly decreased in antibody-treated mice when compared with control group. Distinct CNS pathologies associate with discrete microglia activation modules [44], as increased activation of microglia is found in lesions of MS patients [26,30]. Thus, the decreased expression of these molecules on the innate immune cells in the CNS and reduced LPS-induced IL-6 production in EAE mice receiving anti-CD52-Ab treatment relative to controls indicate a lower activation status of the innate immune cells, which is associated with the significantly reduced CNS inflammation and clinically attenuated EAE symptoms following the antibody treatment [15,28,45].

DCs are the critical player in co-ordinating the immune response during steady state and inflammatory conditions, several studies reported the effect of alemtuzumab on DC population. DCs were shown to be effectively depleted by alemtuzumab both in cultured cells and in patients receiving combined chemoradiotherapy [46] which was followed by its fast reconstitution [47]. However, we consistently observed comparable numbers of circulating and splenic CD11c⁺ DCs between antibody and vehicle-treated EAE mice at first day and eleven days after treatment [15]. The discrepancy of DC number change may be due to the differential expression of CD52 between different subsets of DCs [48,49] studied between different research groups. The number of circulating plasmacytoid

DCs is shown to be reduced in the relapsing and remitting MS patients, but with no change of the surface CD40 and CD80 expression levels at 6 months following alemtuzumab treatment [50]; however, the authors did not perform functional assays to address antigen uptake and presentation capacities of these cells. Our study in the EAE mice suggests an immediate and transit increase in the capacity of peripheral DCs in antigen presentation and thus T-cell stimulation, which was likely associated with the upregulated expression of MHC-II and costimulatory molecules on the cells following the antibody treatment. While no firm conclusion can be drawn yet from the above studies due to the different research conditions, these findings will likely contribute to our understanding of the impact and the underlying immune mechanisms of alemtuzumab treatment for patients.

Surprisingly the immediate phenotypic and functional changes of peripheral innate immune cells observed after anti-CD52-Ab treatment are not maintained in the long term. Increased expression of various important markers on these periphery innate immune cells and associated functions in the anti-CD52-Ab-treated group in comparison with control mice at first day post-injection was not maintained at 3 weeks post-injection. The findings may suggest that the transit phenotypical and functional changes of innate immune cells observed are a compensatory response of the immune system to the anti-CD52-Ab treatment. However, once this is complete, the innate cell repertoire returns to a status similar to the control group rather than experiencing any long-term effects, similar to the fast repopulation of DCs after alemtuzumab treatment in patients [47].

In contrast to the altered response of peripheral innate immune cells between first day and three weeks after treatment, both microglia and infiltrating macrophages in the CNS consistently exhibited similar phenotype and function at both time-points, indicating the unique CNS tissue-specific environment during neuroinflammation. The effect of anti-CD52-Ab on peripheral innate immune cells, but not CNS microglial cells in naïve mice further confirms the association of microglia activation with CNS homeostasis and pathologies. The data would emphasize the importance of considering the different impacts any treatment may have in the periphery and in the CNS when we develop novel therapeutic strategies for neurological diseases.

We also sought to determine whether the changes in innate cell phenotype and function occur only in the presence of an already heightened inflammatory response such as in our EAE model or if the effect would happen regardless of the immune system status upon anti-CD52-Ab administration. Supplementary findings from naïve mice after anti-CD52-Ab treatment revealed similar phenotypic changes of the innate immune cells in the blood and

spleen to that of the EAE mice, both at first day and three weeks after last injection, suggest that these changes are a general response of innate immune cells to anti-CD52-Ab, not specifically associated with host immune conditions. Thus, findings of the effect of anti-CD52-Ab treatment on innate immune cells from this study will not only provide novel insights into the action mechanisms of alemtuzumab treatment in MS [51], but also in its treatment of other diseases such as leukaemia [52] and transplantation [53]. However, it remains elusive what leads to the transit phenotypic and functional changes of peripheral innate immune cells following anti-CD52-Ab treatment. The similar phenotypical changes of blood and spleen DCs and monocytes observed in SCID mice, which are absent of T and B cells, following anti-CD52-Ab treatment suggest that the response of innate immune cells is not a direct result of peripheral mass depletion of lymphocytes.

In conclusion, our study demonstrates a dynamic and tissue-specific modulation of innate DCs, monocytes and microglia in their phenotype and function following treatment with anti-CD52-Ab in EAE mice. The response of the innate immune cells to the antibody is not maintained long term, which may benefit patients by preserving their immunocompetence against infection and other immune-mediated diseases in the long term. Interestingly, the antibody-induced transit response of peripheral innate immune cells is not due to the mass depletion of T and B cells in the periphery, but is likely through some currently unknown mechanisms. Our novel discovery of the differential effect of anti-CD52-Ab on the phenotype and function of microglia and infiltrating macrophages in the CNS in comparison to the cells in the peripheral organs is of particular interest to our understanding of the unique environment of CNS responding to peripheral immune modulation in neuroinflammatory disease. It also supports the beneficial therapeutic effect of alemtuzumab on inhibiting neuroinflammation in MS patients.

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Not applicable.

AUTHOR CONTRIBUTIONS

MB design and perform experiments, collect and analyse data, and prepare paper; RW perform experiments and collect data; TH perform experiments and prepare the paper; TJB analyse data and prepare paper; HRJ design experiments, analyse data and prepare paper.

DATA AVAILABILITY STATEMENT

Raw data were generated at University of Strathclyde. Derived data supporting the findings of this study are available from the corresponding author HRJ request.

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